A. COVER PAGE

Project Title: Understanding the Risk of Bat Coronavirus Emergence	
Grant Number: 5R01AI110964-05	**Project/Grant Period:** 06/01/2014 - 05/31/2019
Reporting Period: 06/01/2018 - 05/31/2019	**Requested Budget Period:** 06/01/2018 - 05/31/2019
Report Term Frequency: Annual	**Date Submitted:** 08/03/2021
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Human Subjects: Yes **HS Exempt:** NA **Exemption Number:** **Phase III Clinical Trial:** NA	**Vertebrate Animals:** NA
hESC: No	**Inventions/Patents:** No

B. ACCOMPLISHMENTS

B.1 WHAT ARE THE MAJOR GOALS OF THE PROJECT?

Zoonotic coronaviruses are a significant threat to global health, as demonstrated with the emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002, and the recent emergence Middle East Respiratory Syndrome (MERS-CoV). The wildlife reservoirs of SARS-CoV were identified by our group as bat species, and since then hundreds of novel bat-CoVs have been discovered (including >260 by our group). These, and other wildlife species, are hunted, traded, butchered and consumed across Asia, creating a largescale human-wildlife interface, and high risk of future emergence of novel CoVs. To understand the risk of zoonotic CoV emergence, we propose to examine 1) the transmission dynamics of bat-CoVs across the human-wildlife interface, and 2) how this process is affected by CoV evolutionary potential, and how it might force CoV evolution. We will assess the nature and frequency of contact among animals and people in two critical human-animal interfaces: live animal markets in China and people who are highly exposed to bats in rural China. In the markets we hypothesize that viral emergence may be accelerated by heightened mixing of host species leading to viral evolution, and high potential for contact with humans. In this study, we propose three specific aims and will screen free ranging and captive bats in China for known and novel coronaviruses; screen people who have high occupational exposure to bats and other wildlife; and examine the genetics and receptor binding properties of novel bat-CoVs we have already identified and those we will discover. We will then use ecological and evolutionary analyses and predictive mathematical models to examine the risk of future bat-CoV spillover to humans. This work will follow 3 specific aims:

Specific Aim 1: Assessment of CoV spillover potential at high risk human-wildlife interfaces. We will examine if: 1) wildlife markets in China provide enhanced capacity for bat-CoVs to infect other hosts, either via evolutionary adaptation or recombination; 2) the import of animals from throughout Southeast Asia introduces a higher genetic diversity of mammalian CoVs in market systems compared to within intact ecosystems of China and Southeast Asia; We will interview people about the nature and frequency of contact with bats and other wildlife; collect blood samples from people highly exposed to wildlife; and collect a full range of clinical samples from bats and other mammals in the wild and in wetmarkets; and screen these for CoVs using serological and molecular assays.

Specific Aim 2: Receptor evolution, host range and predictive modeling of bat-CoV emergence risk. We propose two competing hypotheses: 1) CoV host-range in bats and other mammals is limited by the phylogenetic relatedness of bats and evolutionary conservation of CoV receptors; 2) CoV host-range is limited by geographic and ecological opportunity for contact between species so that the wildlife trade disrupts the ‘natural’ co-phylogeny, facilitates spillover and promotes viral evolution. We will develop CoV phylogenies from sequence data collected previously by our group, and in the proposed study, as well as from Genbank. We will examine co-evolutionary congruence of bat-CoVs and their hosts using both functional (receptor) and neutral genes. We will predict host-range in unsampled species using a generalizable model of host and viral ecological and phylogenetic traits to explain patterns of viral sharing between species. We will test for positive selection in market vs. wild-sampled viruses, and use data to parameterize mathematical models that predict CoV evolutionary and transmission dynamics. We will then examine scenarios of how CoVs with different transmissibility would likely emerge in wildlife markets.

Specific Aim 3: Testing predictions of CoV inter-species transmission. We will test our models of host range (i.e. emergence potential) experimentally using reverse genetics, pseudovirus and receptor binding assays, and virus infection experiments in cell culture and humanized mice. With bat-CoVs that we’ve isolated or sequenced, and using live virus or pseudovirus infection in cells of different origin or expressing different receptor molecules, we will assess potential for each isolated virus and those with receptor binding site sequence, to spill over. We will do this by sequencing the spike (or other receptor binding/fusion) protein genes from all our bat-CoVs, creating mutants to identify how significantly each would need to evolve to use ACE2, CD26/DPP4 (MERS-CoV receptor) or other potential CoV receptors. We will then use receptor-mutant pseudovirus binding assays, in vitro studies in bat, primate, human and other species’ cell lines, and with humanized mice where particularly interesting viruses are identified phylogenetically, or isolated. These tests will provide public health-relevant data, and also iteratively improve our predictive model to better target bat species and CoVs during our field studies to obtain bat-CoV strains of the greatest interest for understanding the mechanisms of cross-species transmission.

B.1.a Have the major goals changed since the initial competing award or previous report?

No

B.2 WHAT WAS ACCOMPLISHED UNDER THESE GOALS?

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B.3 COMPETITIVE REVISIONS/ADMINISTRATIVE SUPPLEMENTS

For this reporting period, is there one or more Revision/Supplement associated with this award for which reporting is required?

No

B.4 WHAT OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT HAS THE PROJECT PROVIDED?

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B.5 HOW HAVE THE RESULTS BEEN DISSEMINATED TO COMMUNITIES OF INTEREST?

1. Conference and University Lectures: PI Daszak and Co-investigators Shi, Epstein, Olival, and Zhang gave invited conference and university lectures at The US-China Dialogue on the Challenges of Emerging Infections, Laboratory Safety and Global Health Security in Galveston, US; the US-China Workshop on Frontiers in Ecology and Evolution of Infectious Diseases in Berkeley, US and Shenzhen, China; the Sino-Germany symposium "Globalization-Challenge and Response for Infectious Diseases" in Hamburg, Germany; the 8th International Symposium on Emerging Viral Diseases in Wuhan, China; the Global Virome Project meeting, Bangkok, Thailand; the Western Asia Bat Research Network (WAB-Net) workshop, Tbilisi, Georgia; the International Conference on Emerging Infectious Diseases (ICEID), Atlanta, US; the North American Society for Bat Research (NASBR) Conference, Puerta Vallerta, Mexico; and the 3rd Symposium of Biodiversity and Health in Southeast Asia, Chiayi, Taiwan

2. Agency and other briefing: PI Daszak and Co-investigators Shi, Olival presented this project at the Cary Institute for Ecosystem Studies, New York, US; the National Institute for Viral Disease Control and Prevention, China CDC; the Chinese Academy of Sciences; and the Chinese Academy of Medical Sciences

3. Public outreach: PI Daszak and Co-investigator Shi, Epstein, Olival, have presented this work to the general public in a series of meetings over Year 5 including at a Cosmos Club briefing that EcoHealth Alliances hosts in Washington DC, multiple meetings of the China National Virome Project and the Global Virome Project in China, Europe, Australia, Southeast Asia and Latin America. As in Year 4, Co-Investigator Zhu introduced this work to the conservation and ecological research community in China through field training workshops.

B.6 WHAT DO YOU PLAN TO DO DURING THE NEXT REPORTING PERIOD TO ACCOMPLISH THE GOALS?

Not Applicable

The results of the 5th year of our R01 work are detailed below. They include:

Specific Aim 1: Assessment of CoV spillover potential at high-risk human-wildlife interfaces

During Year 5, we finalized the analysis of both quantitative and qualitative data from human surveillance in three provinces in Southern China: Yunnan, Guangxi, and Guangdong provinces.

1.1 High-risk human-animal interaction increase bat coronavirus spillover potential among rural residents in southern China

We conducted a cross-sectional biological behavioral surveillance in Yunnan, Guangxi, and Guangdong provinces from 2015 to 2017. From 8 study sites, a total of 1,596 residents were enrolled, of these, 1,585 participants completed the questionnaires and 11 participants withdrew from the questionnaire interview due to personal schedule reasons. After the interviews, 1,497 participants provided biological samples for lab analysis **(Fig. 1)**.

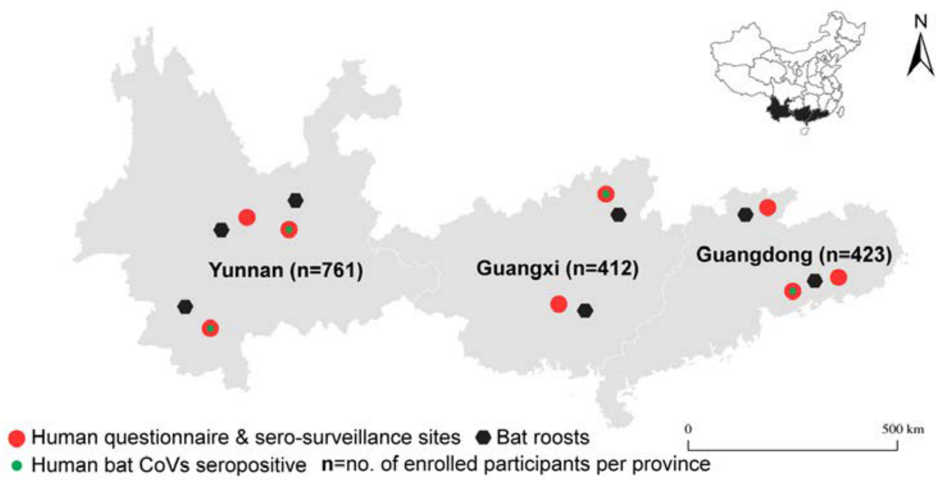


Fig. 1: Eight field surveillance sites for human questionnaire & sero-surveillance with concurrent bat sampling in Yunnan, Guangxi, Guangdong provinces in Southern China. Bat coronavirus sero-positivity were detected in human population in four sites in this study

1.1.1 Demographics

There were more female (62%) than male (38%) from the communities participated in this study. Most participants were adults over 45 years old (69%) and had been living in the community for more than 5 years (97%) with their family members (95%). A majority relied on a comparatively low family annual per capita income less than 10,000 RMB (86%), which is below the national level of per capita disposable income of rural households from 2015 to 2017. Most participants (98%) had not received a higher education from college and were making a living on crop production (76%). 9% of the participants frequently traveled outside the county as migrant laborers.Some participants were working in sectors where frequent human-animal contacts occur, such as the animal production business (1.7%), wild animal trade (0.5%), slaughterhouses or abattoirs (0.5%), protected nature reserve rangers (0.4%) or in wildlife restaurants (0.3%). It was common for participants to have multiple part-time jobs as income sources **(Table 1)**.

Variable	Total	
	N	Valid %
Gender (n= 1,574)		
Fema e	968	61.5
Ma e	605	38.4
Other	1	0.1
Age (n=1,582)		
Under 18 years	71	4.5
18 to 44 years	420	26.5
45 to 64 years	780	49.3
Age 65 or o der	311	19.7
Province (n=1,585)		
Guang Dong	420	26.5
Guang X	412	26.0
Yun Nan	753	47.5
Time of residence (n=1,568)		
< 1 month	4	0.3
1 month – 1 year	12	0.8
1 year – 5 years	26	1.7
> 5 years	1,526	97.3
Family annual per capita income (RMB) (n=1,565)		
<1000	271	17.3
1001-10000	1067	68.2
>10000	227	14.5
Activities to earn livelihood since last year		
Extract on of m nera s, gas, o , t mber (n=1,566)	5	0.3
Crop product on (n=1,569)	1,196	76.2
W d fe restaurant bus ness (n=1,564)	5	0.3
W d/exot c an ma trade/market bus ness (n=1,566)	8	0.5
Rancher/farmer an ma product on bus ness (n=1,566)	27	1.7
Meat process ng, s aughterhouse, abatto r (n=1,567)	8	0.5
Zoo/sanctuary an ma hea th care (n=1,565)	1	0.1
Protected area worker (n=1,567)	7	0.4
Hunter/trapper/f sher (n=1,565)	3	0.2
Forager/gatherer/non-t mber forest product co ector (n=1,566)	4	0.3
M grant aborer (n=1,567)	144	9.2
Nurse, doctor, hea er, commun ty hea th worker (n=1567)	7	0.4
Construct on (n=1,564)	41	2.6
Other (n=1,568)	293	18.7
Highest level of education you completed (n=1,570)		
None	428	27.3
Pr mary Schoo	632	40.3
Secondary schoo /Po ytechn c schoo	479	30.5
Co ege/un vers ty/profess ona	31	2.0
Live with family (n=1,564)		
No	73	4.7
Yes	1491	95.3

Table 1: Demographics of study participants. Total counts differ due to missing responses.

1.1.2 Animal contact and exposure to bat coronaviruses

Serological testing of serum samples from 1,497 local residents revealed 9 individuals (0.6%) were positive for bat coronavirus, indicating exposure at any point in their life to bat-born SARS-related Coronavirus (n=7, Yunnan) and HKU10 Coronavirus (n=2, Guangxi), or other coronaviruses that are phylogenetically closely related to these two coronaviruses (Table 2). All individuals who tested positive (male=6, female=3) were over 45 years old, and most (n=8)

were making a living from crop production.None of those participants reported any symptoms in the preceding 12 months in the interview.

Site	# tested	Bat CoV + (%)	SARSr-CoV Rp3 + (%)	HKU10 + (%)	HKU9 + (%)	MERS-CoV+ (%)
J nn ng, Yunnan	209	6 (2.87)	6 (2.87)	-	-	-
Meng a, Yunnan	168	1 (0.6)	1 (0.6)	-	-	-
J nghong, Yunnan	212	-	-	-	-	-
Lufeng, Yunnan	144	-	-	-	-	-
Guangdong	420	-	-	-	-	-
Guangx	412	2 (0.48)	-	2 (0.48)	-	-

Table 2: ELISA testing of human sera for 4 bat CoVs

Due to the low rate of sero-positivity, we did not conduct statistical comparisons of animal-contact behavior by coronavirus outcome. Figure 2 shows animal contact rates among the survey population (n= 1,585) and among sero-positive individuals (n=9).Participants reported common contact with poultry and rodents/shrews, and most animal contact occurred in domestic settings through raising animal or food preparation activities.

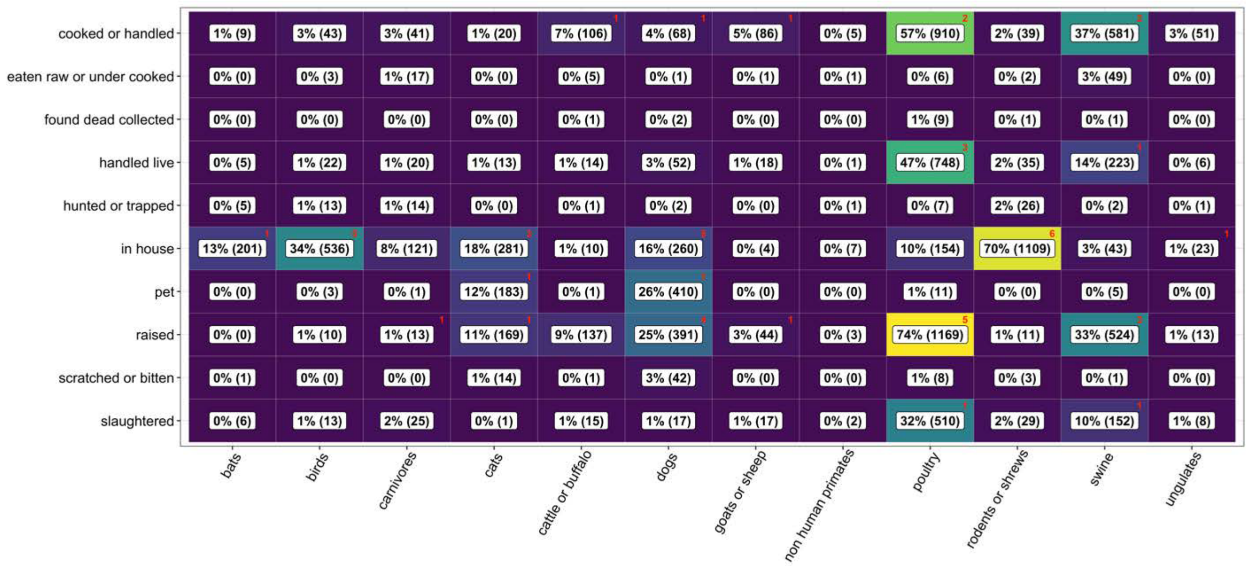


Fig. 2: Animal contact by taxa and activities. Values and shading represent survey population; red numbers in upper-right corners of cells indicate the number of sero-positive individuals with the given contact.

1.1.3 Self-report SARI/ILI symptoms and animal contact

Among the 1,565 participants who responded, 17% (n=265) had experienced fever with cough and shortness of breath or difficulty breathing (38, 14%), indicative of severe acute respiratory infection (SARI), or fever with muscle aches; cough, or sore throat (192, 72%), indicative of influenza like illness (ILI), or both symptoms (35, 13%) in the past 12 months.

LASSO analyses of the associations between animal contact and self-report SARI or ILI symptoms showed that eating raw or undercooked carnivores (OR = 1.6; bootstrap support = 0.67) was the most salient predictor of experiencing SARI or ILI symptoms, followed by slaughtering poultry as a resident of Guangxi province (OR = 1.4; support = 0.68); having an income below 10,000 as a resident of Guangxi province (OR = 1.3; support = 0.84); domestic

contact with bats (OR = 1.3 ; support = 0.63) and domestic contact with rodents or shrews as a resident of Guangdong province (OR = 1.2; support = 0.63) **(Fig. 3)**.

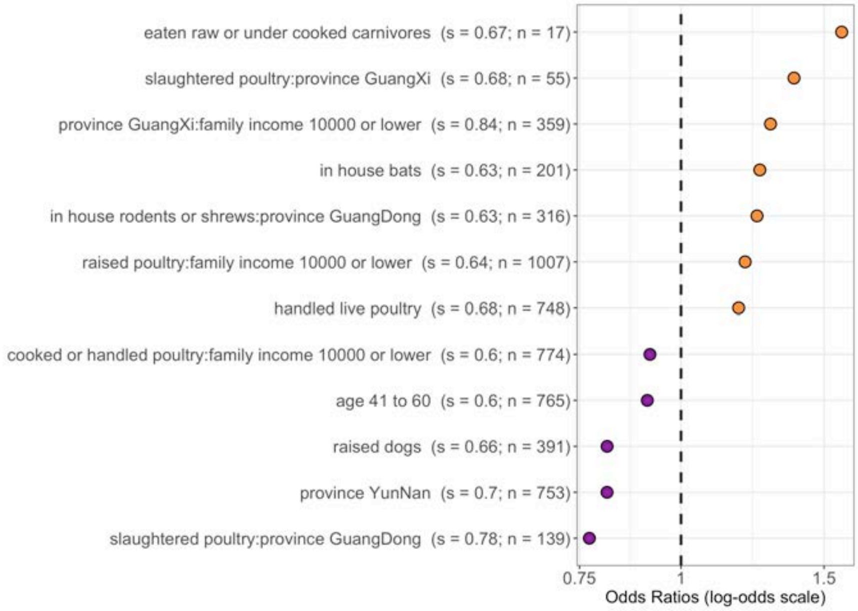


Fig. 3: Most salient predictors of self-reported ILI and/or SARI symptoms in the last year (s = bootstrap support; n = count positive out of 1585 respondents). Bootstrap support values = 0.6 are demonstrated here meaning they were identified as associated with the outcome for 60% or more of the bootstrap iterations. Odds ratios > 1 (orange) are positively associated with the outcome, and odds ratios <1 (purple) are negatively associated with the outcome.

This study provides serological evidence of subclinical or asymptomatic bat-born SARS-related Coronavirus and HKU10 Coronavirus spillover event(s) in rural communities in Southern China, highlights the associations between human-animal interaction and zoonotic spillover risk. The rate of seropositivity observed in this study is clearly lower than would be seen for established human infections. However it has important implications for predicting and preventing pandemics:

1. It indicates that spillover of novel bat-CoVs is detectable if populations that live within areas inhabited by likely bats hosts are targeted. **This provides a pathway to identify spillover events rapidly, perhaps even before a SARS-like disease can become established in people**;
2. It allows us to calculate the likely number of people infected by novel bat SARSr-CoVs annually in this region. Our preliminary analyses suggest that if similar seroprevalence occurs in human populations across the region bat SARSr-CoV hosts inhabit, **there may be as many as the low hundreds of thousands to over a million people infected each year in South China and Southeast Asia.** We aim to conduct a detailed analysis of this in the future.
3. It highlights ways to refine surveillance that could help prevent pandemics, by targeting populations where seroprevalence suggests that they are **at higher risk due to behavioral preferences (e.g. wildlife hunting, farming, or trading)** or where **early-stage SARS-like illnesses could be identified using syndromic surveillance of clinics**.

Contact with poultry and rodents/shrews were commonly reported among participants and associated with self-reported ILI and/or SARI symptoms, which suggests that domestic animals, in addition to wildlife, are an important link in understanding the coronavirus transmission from bat to human populations, indirect exposure might occur through contact with live domestic animals in house or market when the animals had prior exposure to bat coronavirus.

When clinical evidence is limited, undiagnosed or subclinical symptoms similar to SARI and ILI in a population should be brought to our attention as indicators in monitoring zoonotic pathogen spillover events, and considered for prevention strategies. This is particularly important in rural community settings, where people have a higher level of exposure to both domestic and wild animals, but may not seek diagnosis or treatment in a timely fashion, thus slowing the processes of early detection and response.

1.2 Qualitative Approach to Developing Zoonotic Risk Mitigation Strategies in Southern China

To explore the potential drivers of zoonotic exposure and the opportunities for intervention, we conducted field observation and semi-structured ethnographic interviews among 88 community members who have frequent exposure to wildlife and domestic animals and/or have extensive local knowledge in 9 sites in Yunnan, Guangdong, and Guangxi provinces.

The majority of participants in this study were adults between 31 to 50 years of age, residing in rural or suburban areas. Most earned their livelihoods from multiple sources, primarily in crop production, subsistence animal farming, small business, and other temporary jobs as migrant workers. Risk and protective factors were identified at the individual, community, and policy levels regarding potential zoonosis exposures, recommending risk-mitigation strategies with the strengthened policy enforcement and multi-sectoral collaboration among human, animal, and environment health programs **(Fig. 4).**

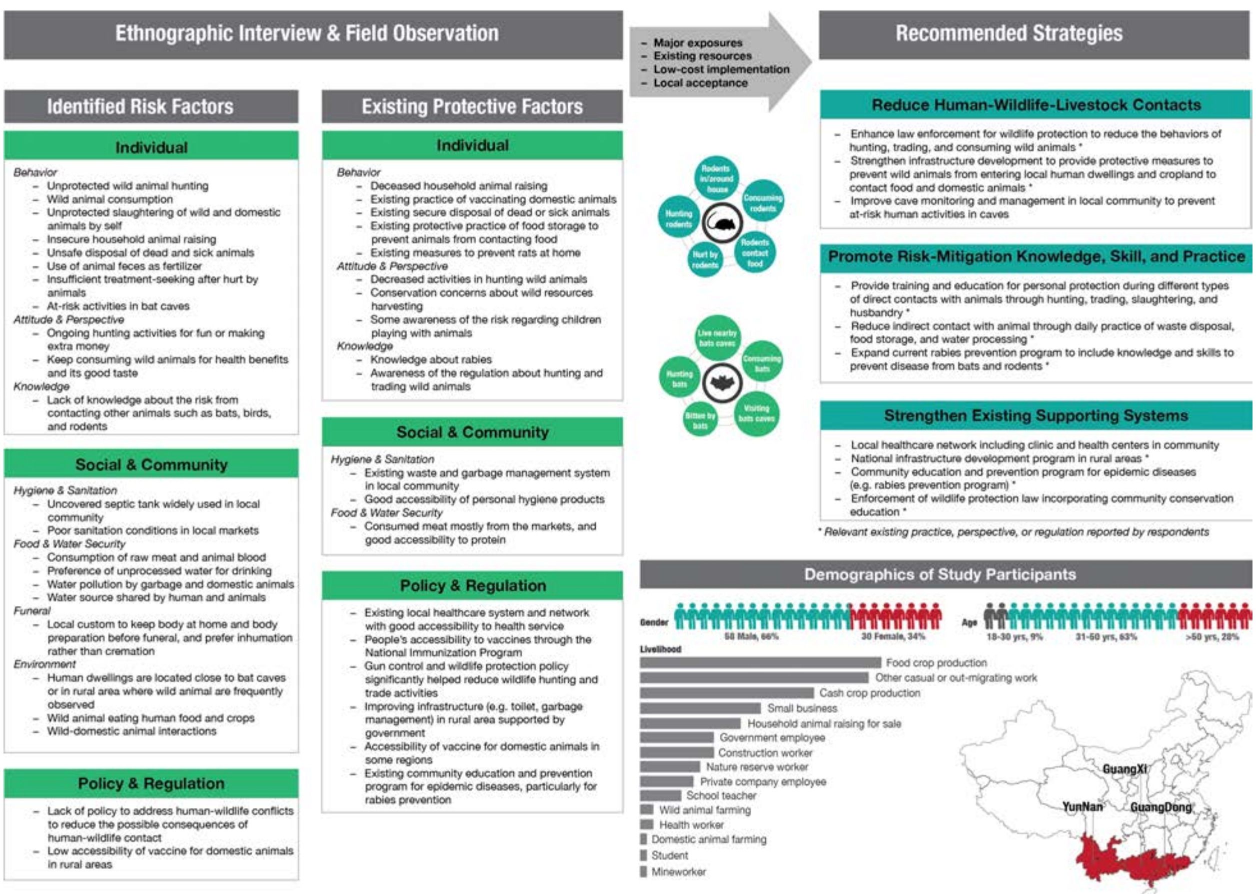


Fig. 1: Community Zoonosis Exposure Risk Mitigation Strategy Development Process. Leveraging ethnographic interview and observational research data to identify risk and protective factors and develop risk-mitigation recommendations

This demonstrated a qualitative approach to understand the zoonotic risks in community, and provided guidance for future research and interventions with focused potential zoonotic risks for disease control and prevention in southern China and a broader area with similar ecological, culture, and demographic contexts.

<u>Specific Aim 2: Receptor evolution, host range and predictive modeling of bat-CoV emergence risk</u>

2.1 Bat CoV PCR detection and sequencing from live-sampled bat populations

From May to October 2018, we collected 1,697 rectal swabs, oral swabs, and feces specimens from 26 bat species in Hubei, Shandong, Yunnan and Guangdong Provinces across southern, central and northern China in Year 5, all specimen were tested for CoV RNA and 109 (6.4%) were positive. SARS-related coronaviruses were discovered in *Rhinolophus sinicus* samples from Yunnan and Hubei provinces while HKU2-related coronaviruses were detected in *R.sinicus* from Hubei. HKU5-related and HKU10-related coronaviruses were identified in *Pipistrellus abramus* from Shandong and *Hipposideros larvatus* from Guangdong, respetively. *Scotophilus* coronavirus 512 was detected in Guangdong. Addtionally, two novel *Pipistrellus* alphacoronaviruses were found in Shandong province in northern China **(Fig. 5)**.

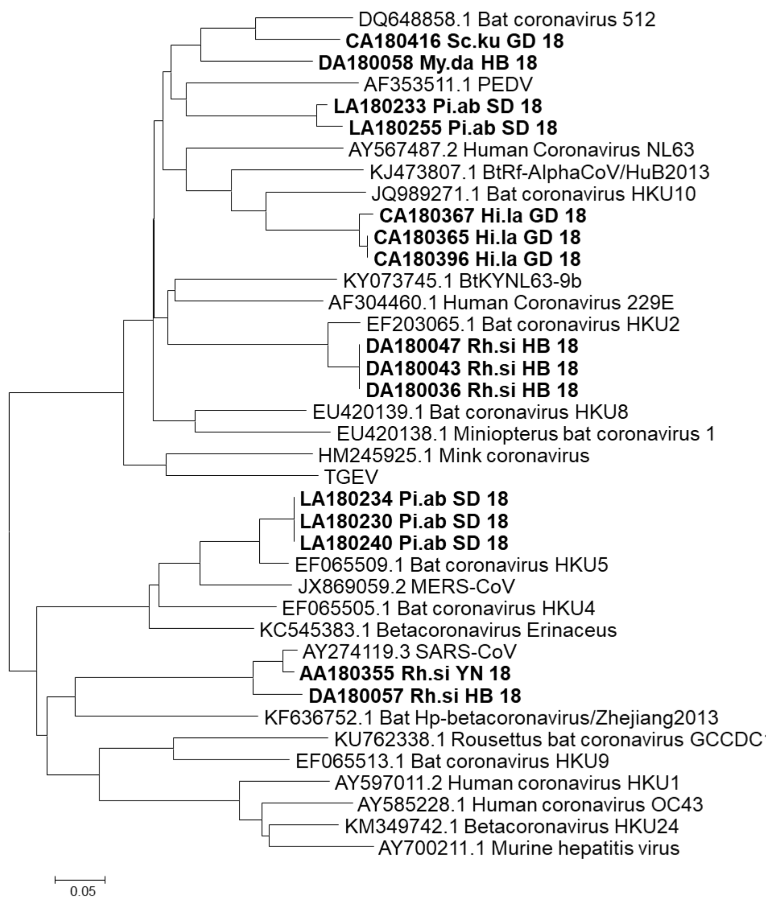


Fig. 2: Phylogenetic analysis of partial RdRp gene of CoV (440-nt partial sequence)

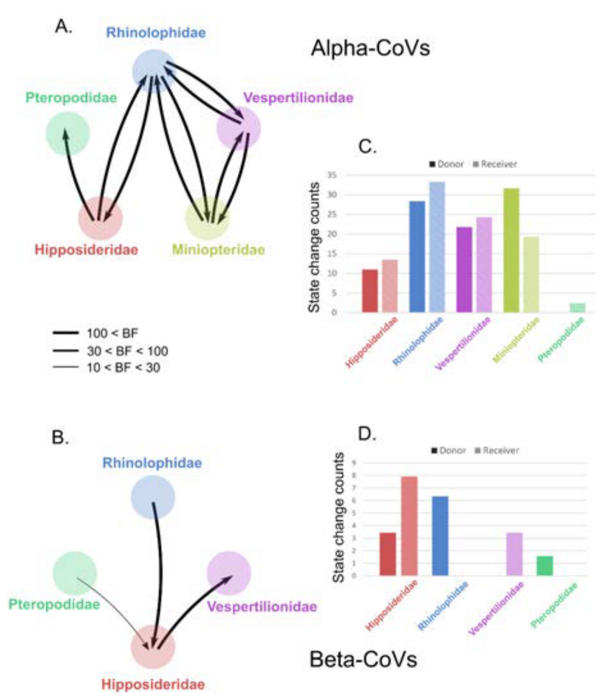
2.2 Bat coronavirus host-virus phylogeography in China

Our dataset includes all CoV RdRp sequences isolated from bat specimens collected by our team from 2008-2015 (Alpha-CoVs: n = 491 – Beta-CoVs: n = 326), including those collected under prior NIAID funding (1 R01 AI079231), and funding from Chinese Federal Agencies. All Chinese bat CoV RdRp sequences available in GenBank were also added to

our dataset (Alpha-CoVs: n = 226 – Beta-CoVs: n = 206). Phylogenetic trees were reconstructed for Alpha- and Beta-CoVs separately using Bayesian inference (BEAST 1.8).

2.2.1 Ancestral hosts and cross-species transmission

We used ancestral character state reconstruction and a Bayesian stochastic search variable selection (BSSVS) to identify host switches between bat families **(Fig. 6)** and genera **(Fig. 7)** that occurred along the branches of the phylogenetic tree and calculated BF to estimate the significance of these non-zero transition rates. We identified nine and three highly supported (BF > 10) **inter-family** host transition rates for alpha- and beta-CoVs, respectively **(Figs. 6A and 6B)**. To quantify the intensity of these host switches, we estimated the number of state changes (Markov jumps) along the significant inter-family transition rates **(Figs. 6C and 6D)**. The total estimated number of inter-family host jump events was more than eight times higher in the evolutionary history of alpha- (n = 90) than beta-CoVs (n = 11) in China. Host transition events from Rhinolophidae and Miniopteridae were greater than from other families for alpha-CoVs while Rhinolophidae were the highest donor family for beta-CoVs. Rhinolophidae and Hipposideridae were the families receiving the highest numbers of transition events for alpha- and beta-CoVs, respectively **(Figs. 6C and 6D)**.



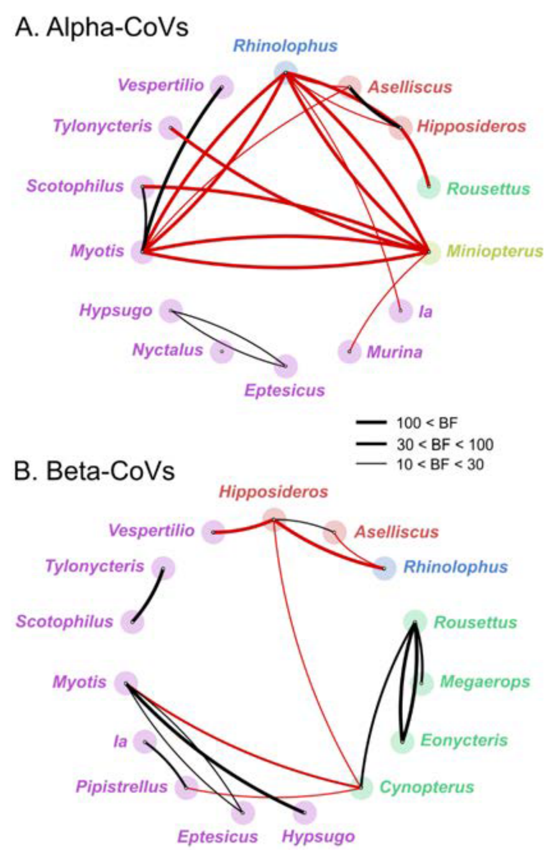


Figure 3: Non-zero transition rates between bat families for alpha- **(A)** and beta-CoVs **(B)** and their significance level (Bayes factor, BF), BF < 10 are considered as non-significant. Arrows indicate the direction of the transition; arrow thickness is proportional to the transition significance level. Histograms show total number of state changes (Markov jumps) from/to each bat family along the significant inter-family transition rates for alpha- **(C)** and beta-CoVs **(D)**.

Figure 4: Non-zero transition rates between bat genera for alpha- **(A)** and beta-CoVs **(B)** and their significance level (Bayes factor, BF), BF < 10 are considered as non-significant. Lines with a rightward curvature depict transitions from that bat genus, while lines with leftward curvature depict transition to that bat genus. Inter-family transitions are highlighted in red.

At the genus level, we identified 20 highly supported inter-genus host transition rates for alpha-CoVs **(Fig. 7A)**. *Rhinolophus* and *Myotis* were the donor genera in four of these transitions while *Miniopterus* and *Rhinolophus* were each the recipients of four of these transitions **(Fig. 7A)**. Sixteen highly supported inter-genus transition rates were identified for beta-CoVs **(Fig.7B)**. Four of these 16 host switches originated in *Cynopterus* while three of them ended in *Myotis* **(Fig. 7B)**. Fifteen out of the 20 significant pairwise host transitions (75%) for alpha-CoVs involved two genera belonging to different bat families, while this proportion is only 6/16 (37.5%) for beta-CoVs. This confirmed the highest number of inter-family host transitions for alpha-CoVs. The estimated total number of inter-genus host switches was almost two times higher for alpha- (n = 123) than beta-CoVs (n = 70).

These findings indicate that alpha-CoVs were able to switch hosts more frequently and between more distantly related taxa during their evolution and suggest that phylogenetic distance among hosts represents higher constraint on host switches for beta- than alpha-CoVs.

2.2.2 CoV spatiotemporal dispersal in China

We also used our Bayesian discrete phylogeographic model using zoogeographic regions as character states to reconstruct the spatiotemporal dynamics of CoV dispersal in China. Eleven and seven highly significant (BF > 10) dispersal routes within China were identified for alpha- and beta-CoVs, respectively **(Fig. 8A and 8B)**. The Rhinacovirus lineage that includes HKU2 and SADS-CoV likely originated in SO region while all other alpha-CoV lineages likely arose in SW China and spread to other regions before several dispersal events occurred from SO and NO in all directions **(Fig. 8A)**.

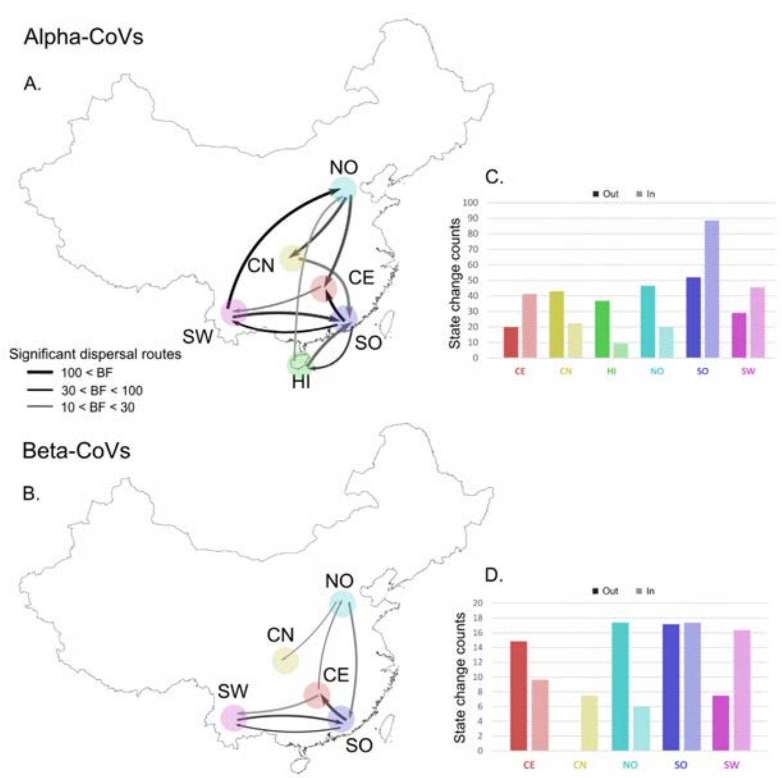


Fig. 8: Significant dispersal routes among China zoogeographic regions for alpha- **(A)** and beta-CoVs **(B)**. Arrows indicate the direction of the transition; arrow thickness is proportional to the transition significance level. Darker arrow colors indicate older dispersal events. **Fig. 8 (C & D)** Histograms of total number of state changes (Markov jumps) from/to each region along the significant dispersal routes for alpha- **(C)** and beta-CoVs **(D)**. NO, Northern region; CN, Central northern region; SW, South western region; CE, Central region; SO, Southern region; HI, Hainan island.

The oldest inferred dispersal movements among beta-CoVs occurred among SO and SW regions **(Fig. 8B)**. SO region is the likely origin of Merbecovirus (Lineage C, including HKU4 and

HKU5) and Sarbecovirus subgenera (Lineage B, including HKU 3 and SARS-related CoVs) while Nobecovirus (lineage D) and Hibecovirus (lineage E) subgenera originated in SW China. Then several dispersal movements likely originated from SO and CE **(Fig. 8B)**. More recent southward dispersal from NO was observed.

The estimated total number of migration events along these significant dispersal routes is four times higher for alpha- (n = 227) than beta-CoVs (n = 57). SO has the highest number of outbound and inbound migration events for alpha-CoVs **(Fig. 8C)**. For beta-CoVs, the highest numbers of outbound migration events have been estimated from NO and SO while SO and SW have the highest numbers of inbound migration events **(Fig. 8D)**.

Our Bayesian ancestral reconstructions revealed the high importance of South western and Southern China as centers of diversification for both alpha- and beta-CoVs. These two regions are clearly hotspots of CoV phylo-diversity, harboring evolutionary old and phylogenetically diverse lineages of alpha- and beta- CoVs.

2.2.3 Phylogenetic diversity

In order to quantitatively evaluate the diversity and the clustering process in our phylogenies, the Mean Phylogenetic Distance (MPD) and the Mean Nearest Taxon Distance (MNTD) statistics and their standardized effect size (SES) were calculated for each zoogeographic region, bat family and genus. The SES corresponds to the difference between the phylogenetic distances in the observed communities versus null communities built by randomly reshuffling tip labels 1000 times along the entire phylogeny. Low and negative SES values denote phylogenetic clustering, high and positive values indicate phylogenetic over-dispersion while values close to 0 show random dispersion.

Significant negative SES MPD values ($p < 0.05$), indicating basal phylogenetic clustering, were observed within all bat families and genera for both alpha- and beta-CoVs, except within *Aselliscus* and *Tylonycteris* for alpha-CoVs **(Figs. 9A & B)**. Negative and mostly significant SES MNTD values, reflecting phylogenetic structure closer to the tips, were also observed within most bat families and genera for alpha- and beta-CoVs but we found non-significant positive SES MNTD value for Vespertilionidae and *Pipistrellus* for beta-CoVs (Fig. 4A and 4B). In general, we observed lower phylogenetic diversity for beta- than alpha-CoVs within all bat families and most genera when looking at SES MPD, while similar level of diversity are observed when looking at SES MNTD **(Figs. 9A & B)**. These results suggest stronger basal clustering (at the deeper nodes) for beta-CoVs than alpha-CoVs.

Chinese zoogeographic regions don’t harbor a random set of CoVs as alpha- and beta-CoV strains within most regions are more closely related than expected by chance as denoted by negative and mostly significant values of MPD and MNTD **(Fig. 9C)**. However, positive SES MPD value for alpha-CoVs in SW indicate wider evolutionary diversity in that region **(Fig. 9C)**.

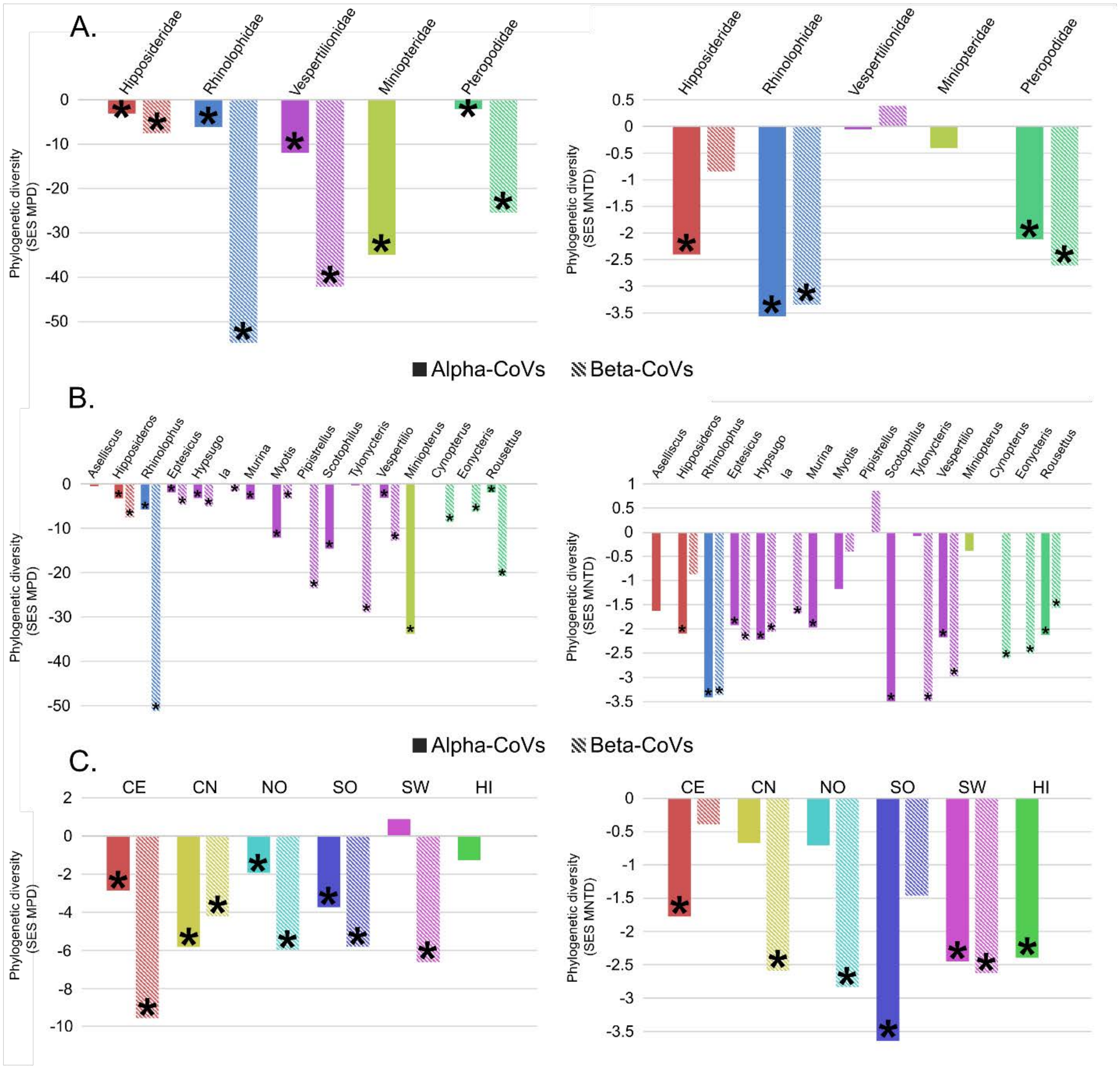


Fig. 9: CoV phylogenetic diversity bat families **(A),** genera **(B),** and zoogeographic regions **(C)**: SES MPD, standardized effect size of Mean Phylogenetic Distance **(Left)**; and SES MNTD, standardized effect size of Mean Nearest Taxon Distance **(Right)**. Values departing significantly from null model (p-value < 0.05) indicated with an asterisk. NO, Northern region; CN, Central northern region; SW, South western region; CE, Central region; SO, Southern region; HI, Hainan island.

2.3 Characterization of SADSr-CoV coronaviruses diversity and distributions

In previous project years, our team identified and characterized Swine Acute Diarrheal Syndrome coronavirus (SADS-CoV), a novel swine virus causing outbreaks in farms in multiple Chinese provinces.In this year, we were able to identify SADS-related CoVs in bats from our wild bat sampling.In >17,000 bat and other mammals at 47 sites across southern China, we found 78 new SADSr-CoVs[11], all in 9 bat species, with mean prevalence of 0.1 to 37.5%.

Our phylogenetic analysis suggests that pig SADS-CoV recently spilled over from *R. sinicus* or *R. affinis* bats **(Fig. 10 Left)** However, analysis of full pig viral genomes from 4 initially infected

farms suggests that either the virus evolved as it circulated or that multiple spillover events occurred **(Fig. 10 Right)**.

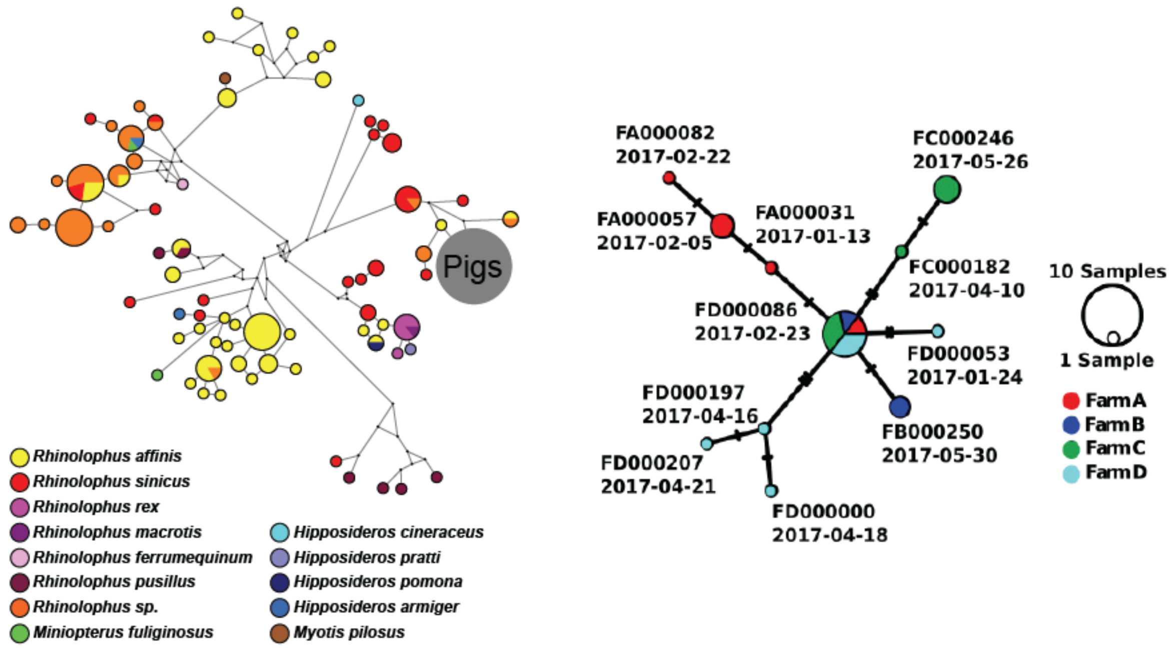


Fig. 10: Left: Median joining network of conserved RdRp gene fragment of 198 unique SADSr-CoV sequences discovered in China under our previous funding. Size of circle proportional to the number specimens with identical viral sequences. **Right:** Median joining network of SADS-CoV full genome sequence data from 4 infected pigs farms in S. China.

We built species distribution models of the major bat species hosts of SADSr-CoVs across southeast Asia to determine the areas where their ranges intersect with large swine operations similar to those of the original outbreak.We found that these are Southern China (including Taiwan), throughout Vietnam, the Philippines, and Thailand. Compared to other countries, China had the largest area of bat-pig overlap with 329,847 km2 (3.4% of total country area) and 2,127,006 pigs located within predicted bat distributions. By Chinese province, the largest area of overlap was found in Jiangsu (35,226 km2 amounting to 34.3% of the province's area and 242,299 pigs within this area). Sichuan had the largest pig population at risk (the pig population within an area that intersects with predicted bat occurrence), at 274,353 heads over 26,015 km2 (5.4% of the total area of the province) **(Figs. 11 & 12)**.

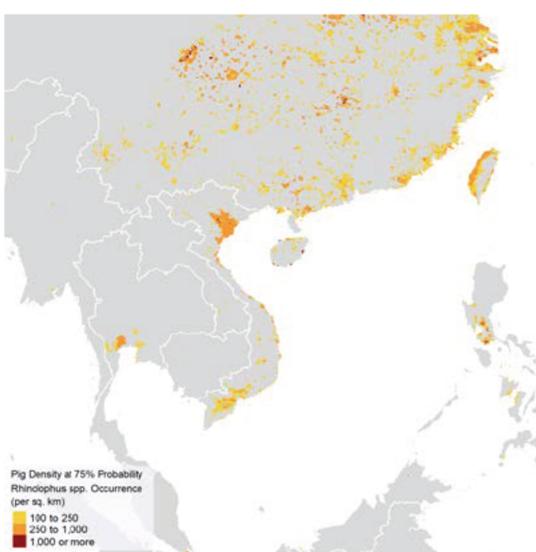


Fig. 5: Areas of bat-pig overlap where probability of SADS-CoV Rhinolophus spp. reservoir occurrence is high (>75%) and pig densities are indicative of intensive pig farming (>100 heads per km2).

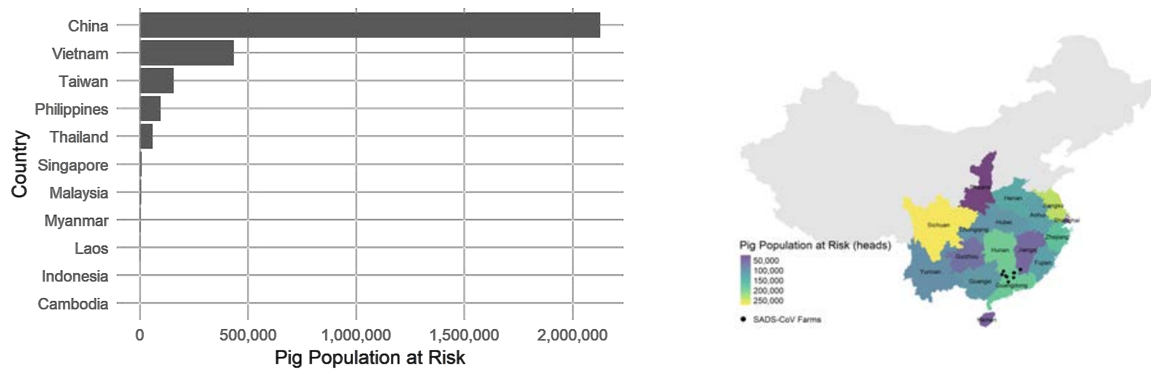


Fig. 6: Top: Country-level, and **Bottom:** province-level estimate of swine populations at-risk based on overlap between modeled populations of bat species known to be SADSr-CoV hosts and large swine operations.

<u>Specific Aim 3: Testing Predictions of CoV Inter-Species Transmission</u>

3.1 *In vivo* infection of Human ACE2 (hACE2) expressing mice with SARSr-CoV S protein variants

In Year 5, we continued with *in vivo* infection experiments of diverse bat SARSr-CoVs on transgenic mice expressing human ACE2. Mice were infected with 4 strains of SARSr-CoVs with different S protein, including the full-length recombinant virus of SARSr-CoV WIV1 and three chimeric viruses with the backbone of WIV1 and S proteins of SHC014, WIV16 and Rs4231, respectively. Pathogenicity of the 4 SARSr-CoVs was evaluated by recording the survival rate of challenged mice in a 2-week course. All of the 4 SARSr-CoVs caused lethal infection in hACE2 transgenic mice, but the mortality rate vary among 4 groups of infected mice **(Fig. 13a)**. 14 days post infection, 5 out of 7 mice infected with WIV1 remained alive (71.4%), while only 2 of 8 mice infected with rWIV1-SHC014 S survived (25%). The survival rate of mice infected with rWIV1-WIV16S and rWIV1-4231S were 50%. Viral replication was confirmed by quantitative PCR in spleen, lung, intestine and brain of infected mice. In brain, rWIV1, rWIV1-WIV16S and rWIV1-4231S cannot be detected 2 days or 4 days post infection. However, rWIV1-SHC014 was detected at all time points and showed an increasing viral titer after infection. The viral load reached more than 10^9 genome copies/g at the dead point **(Fig. 13b)**. We also conducted histopathological section examination in infected mice. Tissue lesion and lymphocytes infiltration can be observed in lung, which is more significant in mice infected with rWIV1-SHC014 S **(Fig. 13d)** than those infected with rWIV1 **(Fig. 13c)**. These results suggest that the pathogenicity of SHC014 is higher than other tested bat SARSr-CoVs in transgenic mice that express hACE2.

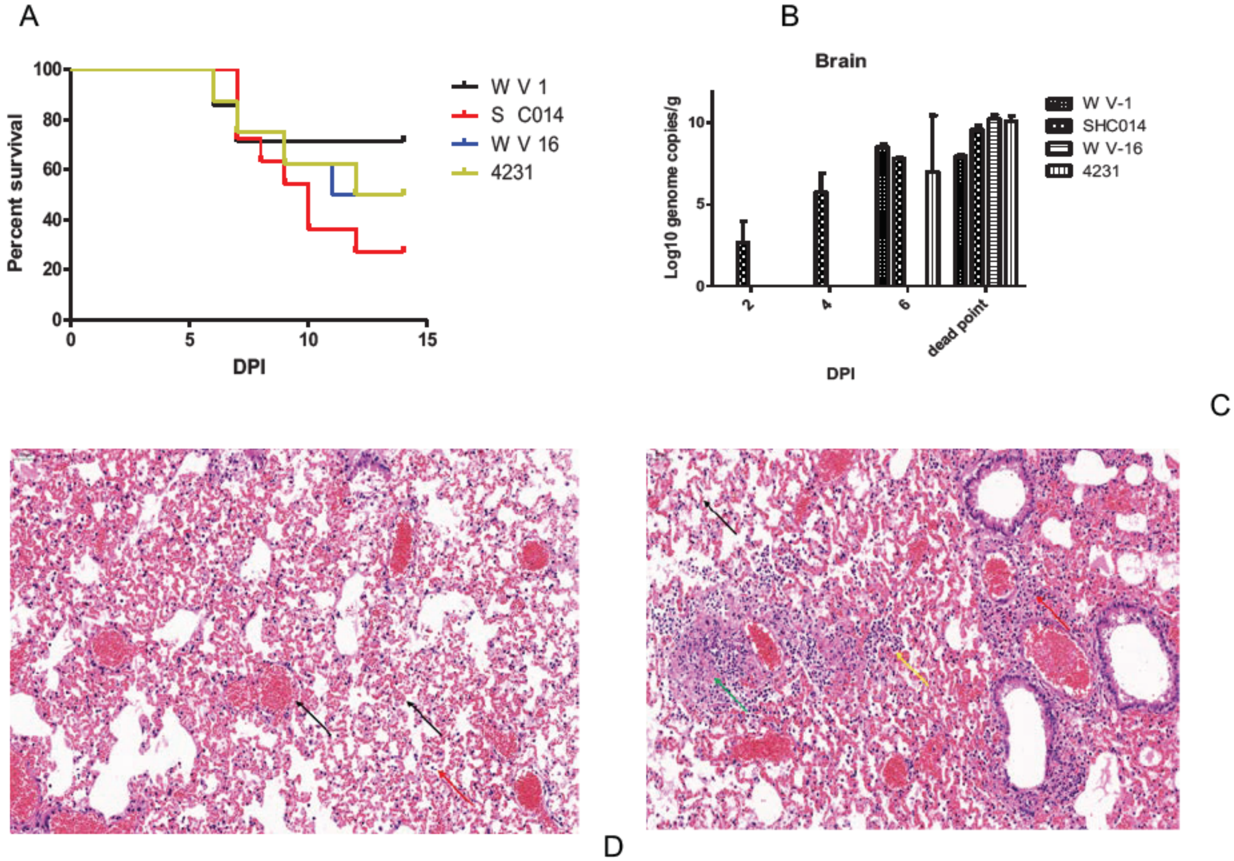


Fig. 13: *In vivo* infection of SARSr-CoV in hACE2-expressing mice. **(A)** Survival rate of hACE2_mice after infection **(B)** Viral load in brains of infected hACE2-expressing mice. **(C)** Histopathological section of lung tissue of mice infected with rWIV1. **(D)** Histopathological section of lung tissue of mice infected with rWIV1-SHC014 S.

3.2 Assessment of interspecies transmission risk of bat HKU4-related coronaviruses

Taking a similar reverse genetics strategy that we used in SARSr-CoV studies, we constructed the full-length infectious clone of MERS-CoV, and replaced the RBD of MERS-CoV with the RBDs of various strains of HKU4-related coronaviruses previously identified in bats from different provinces in southern China. The full-length MERS-CoV and chimeric viruses with RBDs of HKU4r-CoVs were then rescued. Immunofluorescence assay showed that these chimeric MERS-HKU4rRBD coronaviruses were able to infect human cells from different tissues including lung, liver, intestine and kidney **(Fig. 14 Left)**. Moreover, efficient replication of the chimeric HKU4r-CoVs were detected by real-time PCR in HeLa cells that expressed human DPP4 receptor **(Fig. 14 Right)**. The results suggest potential risk of the bat HKU4r-CoVs for cross-species infection in humans.

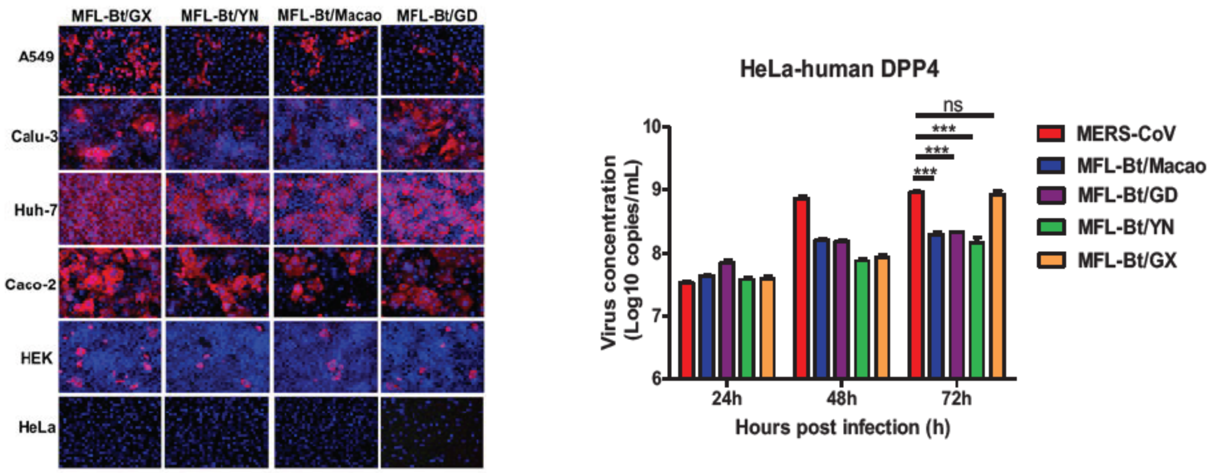


Fig. 7: Left: Immunofluorescence assay confirms Infection of 4 chimeric viruses with the backbone of MERS-CoV and RBD of bat HKU4r-CoVs in different cell lines derived from human tissues. **Right:** Replication of MERS-HKU4rRBD CoVs in HeLa cells expressing human DPP4 was determined by real-time PCR.

B.4 (B4 Training.pdf)

1. Conference and University lectures: We continued to provide human subject research trainings to chief physicians and nurses at local clinics, staff from Yunnan Institute of Endemic Diseases Control and Prevention, students from Dali College and Wuhan University for both qualitative and quantitative research.
2. Agency and other briefing: Dr. Guangjian Zhu provided training to 18 field team members from the Dali College and 4 Wuhan Insitute of Virology laboratory team members regarding biosafety and PPE use, bats and rodents sampling.
3. Public outreach: PI Daszak, and Co-investigators Shi, Epstein, and Olival presented the Year 5 results of this project to the public via interviews with national central and local television, social media, newspaper and journals in China and the US.

C. PRODUCTS

C.1 PUBLICATIONS

Are there publications or manuscripts accepted for publication in a journal or other publication (e.g., book, one-time publication, monograph) during the reporting period resulting directly from this award?

No

C.2 WEBSITE(S) OR OTHER INTERNET SITE(S)

NOTHING TO REPORT

C.3 TECHNOLOGIES OR TECHNIQUES

NOTHING TO REPORT

C.4 INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES

Have inventions, patent applications and/or licenses resulted from the award during the reporting period? No

If yes, has this information been previously provided to the PHS or to the official responsible for patent matters at the grantee organization? No

C.5 OTHER PRODUCTS AND RESOURCE SHARING

NOTHING TO REPORT

D. PARTICIPANTS

D.1 WHAT INDIVIDUALS HAVE WORKED ON THE PROJECT?

Commons ID	S/K	Name	Degree(s)	Role	Cal	Aca	Sum	Foreign Org	Country	SS
(b) (6)	Y	DASZAK, PETER	BS,PHD	PD/PI	(b) (4), (b) (6)					NA
	N	KE, CHANGWEN	PHD	Co-Investigator				Center for Disease Control and Prevention of Guangdon g Province	CHINA	NA
	N	ZHANG, YUNZHI	PHD	Co-Investigator				Yunnan Provincial Institute of Endemic Diseases Control & Prevention	CHINA	NA
	N	ZHU, GUANGJIAN	PHD	Co-Investigator				East China Normal University	CHINA	NA
(b) (6)	N	Chmura, Aleksei	BS,PHD	Non-Student Research Assistant						NA
(b) (6)	N	Ross, Noam Martin	PhD	Co-Investigator						NA
(b) (6)	N	Olival, Kevin J.	PHD	Co-Investigator						NA
(b) (6)	N	Zhang, Shu-yi	PHD	Co-Investigator				East China Normal University	CHINA	NA
(b) (6)	N	SHI, ZHENGLI	PhD	Co-Investigator				Wuhan Institute of Virology	CHINA	NA
	N	GE, XINGYI	PHD	Co-Investigator				Wuhan Institute of Virology	CHINA	NA
(b) (6)	N	EPSTEIN, JONATHAN H	MPH,DVM,BA,PHD	Co-Investigator						NA

Glossary of acronyms:
S/K - Senior/Key
DOB - Date of Birth
Cal - Person Months (Calendar)
Aca - Person Months (Academic)
Sum - Person Months (Summer)
Foreign Org - Foreign Organization Affiliation
SS - Supplement Support
RE - Reentry Supplement
DI - Diversity Supplement
OT - Other
NA - Not Applicable

D.2 PERSONNEL UPDATES

D.2.a Level of Effort

Not Applicable

D.2.b New Senior/Key Personnel

Not Applicable

D.2.c Changes in Other Support

Not Applicable

D.2.d New Other Significant Contributors

Not Applicable

D.2.e Multi-PI (MPI) Leadership Plan

Not Applicable

E. IMPACT

E.1 WHAT IS THE IMPACT ON THE DEVELOPMENT OF HUMAN RESOURCES?

Not Applicable

E.2 WHAT IS THE IMPACT ON PHYSICAL, INSTITUTIONAL, OR INFORMATION RESOURCES THAT FORM INFRASTRUCTURE?

NOTHING TO REPORT

E.3 WHAT IS THE IMPACT ON TECHNOLOGY TRANSFER?

Not Applicable

E.4 WHAT DOLLAR AMOUNT OF THE AWARD'S BUDGET IS BEING SPENT IN FOREIGN COUNTRY(IES)?

Dollar Amount	Country
$66,500	CHINA

G. SPECIAL REPORTING REQUIREMENTS SPECIAL REPORTING REQUIREMENTS

G.1 SPECIAL NOTICE OF AWARD TERMS AND FUNDING OPPORTUNITIES ANNOUNCEMENT REPORTING REQUIREMENTS

NOTHING TO REPORT

G.2 RESPONSIBLE CONDUCT OF RESEARCH

Not Applicable

G.3 MENTOR'S REPORT OR SPONSOR COMMENTS

Not Applicable

G.4 HUMAN SUBJECTS

Sub-Project ID	Study ID	Study Title	Delayed Onset	Clinical Trial	NCT	NIH-Defined Phase 3	ACT
	58010	Understanding the Risk of Bat Coronavirus Emergence-PROTOCOL-001	NO	NO		NO	

G.5 HUMAN SUBJECTS EDUCATION REQUIREMENT

NOT APPLICABLE

G.6 HUMAN EMBRYONIC STEM CELLS (HESCS)

Does this project involve human embryonic stem cells (only hESC lines listed as approved in the NIH Registry may be used in NIH funded research)?

No

G.7 VERTEBRATE ANIMALS

Not Applicable

G.8 PROJECT/PERFORMANCE SITES

Not Applicable

G.9 FOREIGN COMPONENT

Organization Name: Wuhan Institute of Virology
Country: CHINA

Description of Foreign Component:
Principal Laboratory for all Research in China and detailed in our Specific Aims

G.10 ESTIMATED UNOBLIGATED BALANCE

Not Applicable

G.11 PROGRAM INCOME

Not Applicable

G.12 F&A COSTS

Not Applicable

Section 1 - Basic Information (Study 58010)

OMB Number: 0925-0001

Expiration Date: 02/28/2023

1.1. Study Title *

Understanding the Risk of Bat Coronavirus Emergence-PROTOCOL-001

1.2. Is this study exempt from Federal Regulations * ❍ Yes ● No

1.3. Exemption Number ❏ 1 ❏ 2 ❏ 3 ❏ 4 ❏ 5 ❏ 6 ❏ 7 ❏ 8

1.4. Clinical Trial Questionnaire *

1.4.a. Does the study involve human participants?	● Yes	❍ No
1.4.b. Are the participants prospectively assigned to an intervention?	❍ Yes	● No
1.4.c. Is the study designed to evaluate the effect of the intervention on the participants?	❍ Yes	● No
1.4.d. Is the effect that will be evaluated a health-related biomedical or behavioral outcome?	❍ Yes	● No

1.5. Provide the ClinicalTrials.gov Identifier (e.g. NCT87654321) for this trial, if applicable

Section 2 - Study Population Characteristics (Study 58010)

2.1. Conditions or Focus of Study		
2.2. Eligibility Criteria		
2.3. Age Limits	Min Age:	Max Age:
2.3.a. Inclusion of Individuals Across the Lifespan		
2.4. Inclusion of Women and Minorities		
2.5. Recruitment and Retention Plan		
2.6. Recruitment Status	Not yet recruiting	
2.7. Study Timeline		
2.8. Enrollment of First Participant (SEE SECTION 6.3)		

2.9. Inclusion Enrollment Reports

IER ID#	Enrollment Location Type	Enrollment Location
IER 58010	Foreign	

Inclusion Enrollment Report 58010

1. Inclusion Enrollment Report Title* : China Study Report

2. Using an Existing Dataset or Resource* : ❍ Yes ● No

3. Enrollment Location Type* : ❍ Domestic ● Foreign

4. Enrollment Country(ies): CHN: CHINA

5. Enrollment Location(s):

6. Comments:

Planned

Racial Categories	Ethnic Categories				Total
	Not Hispanic or Latino		Hispanic or Latino		
	Female	Male	Female	Male	
American Indian/ Alaska Native	0	0	0	0	0
Asian	1230	1230	0	0	2460
Native Hawaiian or Other Pacific Islander	0	0	0	0	0
Black or African American	0	0	0	0	0
White	0	0	0	0	0
More than One Race	0	0	0	0	0
Total	1230	1230	0	0	2460

Cumulative (Actual)

Racial Categories	Ethnic Categories									Total
	Not Hispanic or Latino			Hispanic or Latino			Unknown/Not Reported Ethnicity			
	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	
American Indian/ Alaska Native	0	0	0	0	0	0	0	0	0	0
Asian	980	616	0	0	0	0	0	0	0	1596
Native Hawaiian or Other Pacific Islander	0	0	0	0	0	0	0	0	0	0
Black or African American	0	0	0	0	0	0	0	0	0	0
White	0	0	0	0	0	0	0	0	0	0
More than One Race	0	0	0	0	0	0	0	0	0	0
Unknown or Not Reported	0	0	0	0	0	0	0	0	0	0
Total	980	616	0	0	0	0	0	0	0	1596

Section 3 - Protection and Monitoring Plans (Study 58010)

3.1. Protection of Human Subjects

3.2. Is this a multi-site study that will use the same protocol to conduct non-exempt human subjects research at more than one domestic site? ❍ Yes ❍ No ❍ N/A

If yes, describe the single IRB plan

3.3. Data and Safety Monitoring Plan

3.4. Will a Data and Safety Monitoring Board be appointed for this study? ❍ Yes ❍ No

3.5. Overall structure of the study team

Section 4 - Protocol Synopsis (Study 58010)

4.1. Study Design

4.1.a. Detailed Description

4.1.b. Primary Purpose

4.1.c. Interventions

Type	Name	Description

4.1.d. Study Phase

Is this an NIH-defined Phase III Clinical Trial? ❍ Yes ● No

4.1.e. Intervention Model

4.1.f. Masking ❍ Yes ❍ No

❏ Participant ❏ Care Provider ❏ Investigator ❏ Outcomes Assessor

4.1.g. Allocation

4.2. Outcome Measures

Type	Name	Time Frame	Brief Description

4.3. Statistical Design and Power

4.4. Subject Participation Duration

4.5. Will the study use an FDA-regulated intervention? ❍ Yes ❍ No

4.5.a. If yes, describe the availability of Investigational Product (IP) and Investigational New Drug (IND)/ Investigational Device Exemption (IDE) status

4.6. Is this an applicable clinical trial under FDAAA? (SEE SECTION 6.6)

4.7. Dissemination Plan

I. OUTCOMES

I.1 What were the outcomes of the award?

The aims of our grant (R01AI110964) were to: 1) Analyze the risk that there could be a repeat of the SARS outbreak, due to bat coronaviruses still circulating in China; 2) Work out how we can predict which bat viruses would be most likely to emerge, so that we can prevent new outbreaks; 3) Using lab tests, find out if any of the coronaviruses still present in bat populations in China have the potential to infect people. The overall goal of this work is to help design vaccines and therapeutics against future potentially emerging viruses, work out which communities are on the frontline of a new potential outbreak, and reduce the risk of them being infected by analyzing their risk behavior. During this 5-year period of work, we made significant discoveries leading to 18 peer-reviewed scientific papers, including in some of the world's foremost scientific journals.

Overall, our work shows that bats in China harbor a high number and diversity of coronaviruses, some closely related to SARS-CoV (the virus that caused the SARS pandemic in 2003). We sampled over 16,000 individual bats and found evidence of hundreds of different SARS-related coronavirus genetic sequences. We found out that bats across China harbor these viruses, and that they are common, with 6.7% of bats sampled being positive. Many of these bats are found across China, Southeast Asia, South Asia and beyond, suggesting viruses with zoonotic potential may exist in those regions also. Many of these bats are abundant, and roost and feed close to people and livestock, suggesting high potential for future viral spillover. We also dentified one cave system in Yunnan Province with horseshoe bats that had diverse SARSr-CoVs, including some with S proteins able to use human ACE2 as entry receptors. Bats in this cave carried SARSr-CoVs with all unique genetic elements of the SARS-CoV outbreak virus, suggesting that this site may be a potential public health risk.

To analyze which viruses were a potential public health risk, we managed to culture three strains of SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874. We used the genetic codes of some of the other viruses we found in bats and inserted the spike protein genes of those viruses (the proteins that attach to cells) into the cultured viruses. By doing this experiment we showed that other viruses may also be able to infect human cells, and were able to do this safely without the need to culture large amounts of virus. We also showed that some of these viruses cause SARS-like illness in mice that are adapted to have similar cell surface receptors to people. This work proves that there is a clear and present danger for future emergence of novel SARS-like viruses in people. We also demonstrated that outbreaks can happen in livestock. In 2016-17, we analyzed fecal samples from pigs at 5 farms in South China affected by a fatal diarrheal disease. We discovered a new coronavirus, Swine Acute Diarrheal Syndrome coronavirus (SADS-CoV), and showed that it originates in bats, caused the death of more than 20,000 pigs, but also is able to infect human cells in the lab.

Our work has produced predictive algorithms to map hotspots of viral risk so that public health measures can be taken to protect communities at the frontline of potentially the next SARS pandemic. We have produced new reagents and viral cultures that can be used by labs across the world to design novel vaccines and therapeutics against SARS-CoV and other related viruses that might emerge in the future. Finally, our work has been used directly by the WHO to list SARS-related coronaviruses as one of the highest priority group of pathogens with pandemic potential, so that efforts can be taken to stop a future pandemic before it happens.

Inclusion Enrollment Report

Inclusion Data Record (IDR) #: 166195

Using an Existing Dataset or Resource: No

Delayed Onset Study ?: No

Clinical Trial: No

Enrollment Location: Foreign

NIH Defined Phase III Clinical Trial: No

Study Title: Understanding the Risk of Bat Coronavirus Emergence-PROTOCOL-001

Planned Enrollment

Racial Categories	Ethnic Categories									Total
	Not Hispanic or Latino			Hispanic or Latino			Unknown/Not Reported Ethnicity			
	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	
American Indian/Alaska Native	0	0		0	0					**0**
Asian	1230	1230		0	0					**2460**
Native Hawaiian or Other Pacific Islander	0	0		0	0					**0**
Black or African American	0	0		0	0					**0**
White	0	0		0	0					**0**
More than One Race	0	0		0	0					**0**
Unknown or Not Reported										
Total	**1230**	**1230**		**0**	**0**					**2460**

Cumulative Enrollment

Racial Categories	Ethnic Categories									Total
	Not Hispanic or Latino			Hispanic or Latino			Unknown/Not Reported Ethnicity			
	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	
American Indian/Alaska Native	0	0	0	0	0	0	0	0	0	**0**
Asian	980	616	0	0	0	0	0	0	0	**1596**
Native Hawaiian or Other Pacific Islander	0	0	0	0	0	0	0	0	0	**0**
Black or African American	0	0	0	0	0	0	0	0	0	**0**
White	0	0	0	0	0	0	0	0	0	**0**
More than One Race	0	0	0	0	0	0	0	0	0	**0**
Unknown or Not Reported	0	0	0	0	0	0	0	0	0	**0**
Total	**980**	**616**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1596**